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Streptomyces* sp. AT37 isolated from a Saharan soil produces a furanone derivative active against multidrug-resistant *Staphylococcus aureus

El Hadj Driche^{1,2} · Nasserline Sabaou¹ · Christian Bijani³ · Abdelghani Zitouni¹ · Frédéric Pont⁴ · Florence Mathieu⁵ · Boubekeur Badji¹

Abstract A novel actinobacterium strain, named AT37, showed a strong activity against some multidrug-resistant *Staphylococcus aureus*, including methicillin-resistant *S. aureus* MRSA ATCC 43300, other clinical isolates of MRSA and vancomycin resistant *S. aureus* VRSA S1. The strain AT37 was isolated from a Saharan soil by a dilution agar plating method using chitin-vitamin agar medium supplemented with rifampicin. The morphological and chemical studies indicated that this strain belonged to the genus *Streptomyces*. Its 16S rRNA gene sequence was determined and a database search indicated that it was closely associated with the type strain of *Streptomyces novaecaesareae* NBRC 13368^T with 99.6% of similarity. However,

the comparison of the morphological and the physiological characteristics of the strain with those of the nearest species showed significant differences. The strain AT37 secreted the antibiotic optimally during mid-stationary phase of growth. One active compound (AT37-1) was extracted from the culture broth with dichloromethane, separated on silica gel plates and purified by HPLC. Based on spectroscopic analysis of UV–Visible, infrared, and ¹H and ¹³C NMR spectra and spectrometric analysis, the chemical structure of the compound AT37-1 was identified as 5-[(5E,7E,11E)-2,10-dihydroxy-9,11-dimethyl-5,7,11-tridecatrien-1-yl]-2-hydroxy-2-(1-hydroxyethyl)-4-methyl-3(2H)-furanone. Minimum inhibitory concentrations and minimum biofilm inhibitory concentration (MBIC₅₀) of this compound showed significant activity against multidrug-resistant *S. aureus* with 15–30 and 10–15 µg/mL, respectively.

✉ Nasserline Sabaou
sabaou@yahoo.fr

✉ Boubekeur Badji
badji_boubekeur@yahoo.fr

¹ Laboratoire de Biologie des Systèmes Microbiens (LBSM), Ecole Normale Supérieure, Kouba, Alger, Algeria

² Département de Biologie, Faculté des Sciences de la Nature et de la Vie, Université Hassiba Benbouali de Chlef, Hay Salem, Chlef 02000, Algeria

³ Laboratoire de chimie de coordination (LCC), CNRS, Université de Toulouse, UPS, INPT, LCC, 205, route de Narbonne, 31077 Toulouse, France

⁴ Proteomics Group, INSERM UMR1037, Centre de Recherches en Cancérologie de Toulouse (CRCT), Toulouse, France

⁵ Laboratoire de Génie Chimique, LGC, UMR 5503 (CNRS/INPT/UPS), Université de Toulouse, Toulouse, France

Keywords *Streptomyces* · Saharan soil · Furanone derivative · Antibiofilm · Multidrug-resistant *Staphylococcus aureus*

Introduction

The emergence of resistant bacteria to commercially available antibiotics is a major problem that continues to challenge the healthcare sector (Morel and Mossialos 2010). The increase of the drug resistance in bacteria could be to several factors including the unrestricted and overuse of antibiotics in treatment of infections and food animals (Gravel et al. 2011) and rapid transmission of horizontal and vertical resistance genes between bacteria (Jensen and Lyon 2009). Among the major multidrug-resistant bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA)

is the most Gram positive causing important community-acquired (CA-MRSA) and hospital-acquired infections (HA-MRSA) and also with low degree, some strains of vancomycin-resistant *S. aureus* (VRSA) (Rebiahia et al. 2011). Both CA-MRSA and HA-MRSA are resistant to traditional antistaphylococcal β -lactam antibiotics, but HA-MRSA strains are resistant to several family of antibiotics such as aminoglycosides, macrolides, fluoroquinolones, tetracyclines and glycopeptides (Sharma et al. 2014). MRSA is the causal agent of multiple nosocomial infections worldwide, including carbuncles, sore throat, skin infections, endocarditis, meningitis, sepsis, food poisoning, toxic shock syndrome, osteomyelitis, surgical wound and deep-tissue ulcers catheter-associated bacteremia in hemodialysis patients (Naik and Deshpande 2011). In fact, multidrug-resistant *S. aureus* used generally three great types of resistance mechanisms including enzymatic inactivation of the antibiotic (penicillinase and aminoglycoside-modification enzymes), alteration of the target with decreased affinity for the antibiotic (notable examples being penicillin-binding protein 2a of methicillin-resistant *S. aureus* and D-alanine-D-lactate of peptidoglycan precursors of vancomycin-resistant strains) and efflux pumps (fluoroquinolones and tetracycline) (Pantosti et al. 2007). MRSA is also known to form biofilms on an implanted medical device or damaged tissues, causing several infections which are difficult to treat due to their inherent antibiotic resistance (Kwiecinski et al. 2016).

Several works in the world have reported the occurrence of new strains of *S. aureus* resistant to several antibiotics, specially methicillin and vancomycin (Von Nussbaum et al. 2006; Naik and Deshpande 2011). Therefore, it is imperative to search for new and effective antistaphylococci with antibiofilm activity to combat the menace of drug-resistant infections. Four strategies have been employed: first, the preparation of synthetic antibiotics such as diarylquinolones (Andries et al. 2005); second, the semi synthetic of the natural compounds to create successive generations of antibiotics (tigecycline, a third-generation semi-synthetic tetracycline antibiotic) (Von Nussbaum et al. 2006); third, the search for novel targets within the bacteria (Coates and Hu 2007); fourth, the isolation of new molecules from natural sources (cultured and uncultured microbes) with antibacterial and antibiofilm activities (Clardy et al. 2006; Nithya et al. 2011). We are interested in this work to the latter alternative.

Naturally occurring antibiotics are largely produced by actinobacteria. These microorganisms are filamentous Gram-positive bacteria with high G+C content, and are the most widely distributed in nature which primarily inhabits the soils (Goodfellow et al. 2012). Among them, the genus *Streptomyces* is a predominant component of the microbial population in soils and has been the subject

of extensive isolation and screening efforts over the years because it is a major source of commercially and therapeutically important secondary metabolites (Bérdy 2012). It has been estimated that approximately 45% of natural antibiotics were isolated from actinobacteria, and about 75% of bioactive molecules coming from actinobacteria are produced by members of the genus *Streptomyces* (Solecka et al. 2012). Among the natural antibiotics, the furanones and their derivatives showed an interesting activity against *S. aureus* and other Gram-positive bacteria. These compounds are widely investigated for antibacterial effect, inhibition of biofilm formation, and improvement of antibiotics efficacy (Hentzer et al. 2002; Lonn-Stensrud et al. 2009; Kuehl et al. 2009; Brackman and Coenye 2015; Trizna et al. 2016). Several works showed that the Saharan soils are rich in actinobacteria (Sabaou et al. 1998; Meklat et al. 2013). Some of these actinobacteria secreted interesting antimicrobial substances (Boubetra et al. 2013a; Yekkour et al. 2015) and some of them are new species (Boubetra et al. 2013b; Meklat et al. 2013).

The aim of this study is to describe the isolation and identification of a *Streptomyces* strain designated AT37 by polyphasic approach, and also the production, the purification and the characterization of its antibiotic exhibiting activity against multidrug-resistant *S. aureus*.

Materials and methods

Isolation of the actinobacterium strain

The actinobacterium strain AT37 was isolated from a Saharan soil sample collected in Adrar (South-West of Algeria, 27°51'N, 0°19'W). The soil sample was subjected to serial dilutions in sterile deionized water and diluted. Aliquots (0.2 mL) of each dilution were spread onto chitin-vitamin agar medium, recommended by Hayakawa and Nonomura (Hayakawa and Nonomura 1987) for isolation of actinobacteria. The medium was supplemented with rifampicin (25 μ g/mL) and cycloheximide (80 μ g/mL) to inhibit the growth of bacteria and fungi, respectively. The plates were incubated at 30°C for 2 weeks and then examined by light microscopy. After isolation, the strain AT37 was stored on agar slant of ISP2 medium (Shirling and Gottlieb 1966) at 4°C.

Identification of the actinobacterium strain

Morphological and chemotaxonomic studies

The genus of the selected isolate was initially determined by morphological features and chemical analysis of cellular constituents. The cultural characteristics of the strain were

observed by naked-eye examination of 14 day-old cultures grown on various International *Streptomyces* Project (ISP) media: yeast extract–malt extract agar (ISP2), oatmeal agar (ISP3), inorganic salts–starch agar (ISP4) (Shirling and Gottlieb 1966), and also on Bennett medium (Jones 1949) and nutrient agar medium. The micromorphology and sporulation were observed by light microscopy. Colors of aerial and substrate mycelia were determined with the ISCC-NBS color charts (Kelly and Judd 1976).

For the chemotaxonomic analysis, strain AT37 was grown on ISP2 broth at 30 °C for 4 days on a rotary shaker (250 rpm). Biomass was harvested by centrifugation at 3500 rpm and washed several times with distilled water. Analysis of diaminopimelic acid and whole-cell sugars was carried out using the methods of Becker et al. (1964) and Lechevalier and Lechevalier (1970). Phospholipids were analyzed according to the procedures developed by Minnikin et al. (1977).

Physiological characteristics

Sixty-one physiological tests were used to characterize the actinobacterium strain as described by Locci (1989). They include the utilization of 20 carbohydrate compounds, the degradation of 8 organic compounds, the decarboxylation of 7 organic acids, the assimilation of 6 amino acids, the reduction of nitrate, the sensitivity to phenol (0.001%, w/v), sodium azide (0.001%, w/v), sodium chloride (4 and 7%, w/v) and crystal violet (0.05%, w/v), the growth at pH 5, 7 and 9, at 30, 40 and 45 °C, and also in the presence of 7 antibiotics. The production of melanoid pigments was tested on peptone–yeast extract–iron agar (ISP6) and tyrosine agar (ISP7) media (Shirling and Gottlieb 1966).

Amplification and 16S rRNA gene analysis

PCR amplification and sequence analysis were realized according to the method of Liu et al. (2000). The strain AT37 was grown at 30 °C for 5 days in a shake flask containing 100 mL of ISP2 medium. The 16S rRNA gene was amplified by PCR using two primers: 27f (50-AGA GTTTGATCCTGGCTCAG-30) and 1492r (50-GGT TACCTTGTTACGACTT-30). The final 50- μ L volume of reaction mixture contained 1 \times PCR buffer (10 Mm Tris–HCl, 50 mM KCl, pH 9.0 at 25 °C), 1.5 mM of MgCl₂, 200 μ M of each dNTP, 1 μ M of each primer, 1.25 U of *Taq* DNA polymerase and 1 μ L (500 ng) of the purified DNA. The amplification was performed on a Bio-rad MyCycler according to the following profile: an initial denaturation step at 98 °C for 3 min, after which, *Taq* polymerase was added, followed by 30 amplification cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 2 min, and a final extension step of 72 °C for 10 min. The

PCR product was detected by agarose gel electrophoresis and was visualized by ultraviolet (UV) fluorescence after GelRed Biorad staining. The sequencing reaction was performed by the Beckman Company (Takeley, United Kingdom).

Phylogenetic analysis

The sequence obtained was compared for similarity level with available sequences of the reference species of bacteria contained in the GenBank database, using the EzTaxon-e server (Kim et al. 2012). The phylogenetic analyses were conducted using the Molecular Evolution Genetics Analysis (MEGA) software version 5.0 (Tamura et al. 2011). The 16S rRNA gene sequence of the strain AT37 was aligned using the CLUSTAL W program (Thompson et al. 1994) against corresponding nucleotide sequences of representatives of the genus *Streptomyces* retrieved from GenBank database. The evolutionary distance matrices were generated as described by Kimura (1980) and a phylogenetic tree was constructed via the neighbor-joining algorithm (Saitou and Nei 1987). The tree topologies were evaluated by bootstrap analysis (Felsenstein 1985) using 1000 resamplings.

Antistaphylococcal activity of the strain on solid medium

The antistaphylococcal activity of the strain AT37 was evaluated by the cross-streak plate method against various strains of *S. aureus*. A single streak of actinobacterial culture was streaked at the corner of the Petri plates containing Bennett medium and incubated at 30 °C for 8 days. After growth of strain, target staphylococci were seeded in streaks crossing the actinobacterium culture. The antistaphylococcal activity was appreciated by measuring the length of inhibition between the target microorganisms and the actinobacteria colony margins after incubation at 30 °C for 24 h. Seven target staphylococci were tested (Table 2), and chosen for their sensitivity and resistance to methicillin and others antibiotics. Except strains with ATCC numbers, all other target staphylococci were isolated from diseased patients undergoing antibiotic therapy, from medical equipment and from hospitals environment sampled in three Algerian hospitals located in Algiers. They include *S. aureus* 38, *S. aureus* 39, *S. aureus* 636 and *S. aureus* S1, *S. aureus* R2 and *S. aureus* MRSA ATCC 43300 (multidrug-resistant to antibiotics), and *S. aureus* ATCC 25923 (methicillin sensitive). The assay was performed in triplicate, and results were expressed as mean with the standard deviation.

Time course of growth and antistaphylococcal compound production

Fermentation was carried out in Bennett broth medium for 10 days in order to select the culture time favorable for antibiotic production. The pH of the medium was adjusted to 7.2 prior to autoclaving. The 500-mL Erlenmeyer flasks containing 100 mL of medium was inoculated with 5% (v/v) of the actinobacterium culture grown on medium for 72 h at 30°C. The Erlenmeyer flasks were incubated at 30°C for 10 days with shaking at 250 rpm. The antistaphylococcal activity was measured by well diffusion method against methicillin resistant *S. aureus* (MRSA) ATCC 43300. Each well of 10 mm in diameter was filled with 0.2 mL of supernatant. The growth (dry weight of mycelium) and the pH were also examined daily.

Extraction and antistaphylococcal assay

In order to choose the best solvent of extraction, the bioactive compounds were extracted by four organic solvents with different polarity on the day of optimal production rate. The fermented culture broth of the strain AT37 was centrifuged at 5000 rpm for 10 min to remove the biomass. 50 mL of the cell free supernatant was extracted with an equal volume of the following organic solvents: *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol. The organic layer was dehydrated with Na₂SO₄ and concentrated using the rotavapor. The resulting dry extract was recuperated in 0.5 mL methanol and bioassayed against *S. aureus* MRSA ATCC 43300 by the paper disk diffusion method. The suspension of target microorganism used was adjusted to 10⁶ CFU/mL and swabbed over Muller-Hinton agar. 30 µL of solvent extracts was added to each disk and sterilized under UV light (254 nm) for 45 min. After 24 h of incubation at 37°C, the diameter of the zone of inhibition was measured to evaluate the antibacterial assay of the strain AT37. The experiment was performed with three replicates, and the mean values along with the standard deviation of the concerned data (*n*=3) are presented in the results.

Production and purification of antistaphylococcal compound

The production of antistaphylococcal compound was carried out in 500-mL Erlenmeyer flasks containing 100 mL of Bennett broth medium. The cultures were incubated on a rotary shaker (250 rpm) at 30°C for the day of optimal production rate. The Bennett culture broth was centrifuged to remove the biomass and extracted by the best solvent of extraction.

The bioautography was realized on thin-layer chromatography silica gel plate (Merck Art. 5735, Kiesselgel

60HF 254–366; 20×20 cm) for the partial purification of antistaphylococcal compound. The crude extract was dissolved in methanol, spotted and developed in the solvent system ethyl acetate–methanol (100:15 v/v). The developed TLC plates were air dried overnight to remove all traces of solvents. The separated compounds were visualized under UV at 254 and 365 nm, and the active spot was detected by bioautography and revealed by chemical reagents. The TLC plates were deposited in a plastic bioassay dish (23×23×2.2 cm, Fisher Scientific Labosi, Elancourt, France) and overlaid with 50 mL (per plate) Muller-Hinton medium (containing 7 g/L agar) seeded with *S. aureus* MRSA ATCC 43300 as target microorganism, and incubated at 30°C for 24 h. The location of the bioactive compound and its retention factor (Rf) were detected and measured by observing a clear area that indicate the inhibition of the growth of the target microorganism.

The crude extract was finally purified by Agilent reverse HPLC under the following conditions: flow, 1.2 mL/min; stationary phase, ZORBAX SD C18 column (9.4×250 mm, 5 µm), pre-column, (10×10 mm, 5 µm); mobile phase, a continuous linear gradient solvent system from 10 to 100% methanol in bi-distilled water for 40 min; DAD detector wavelength, 220 nm. All the peak fractions were collected and tested against *S. aureus* MRSA ATCC 43300. The final purification was achieved after the second re-injection in HPLC system.

Spectroscopic analysis and structure elucidation of active compound AT37-1

The UV absorption spectrum of the active compound in methanol was determined with a Shimadzu UV 1605 spectrophotometer. The infrared (IR) spectrum was measured on a Shimadzu IR 470 using KBr tablets. The mass spectrum was recorded on an LCQ ion-trap mass spectrometer (Finnigan MAT, San Jose, CA) with nanospray ion electro-spray ionization (ESI) source (positive and negative ion modes). ¹H and ¹³C NMR spectroscopy were used for the characterization of AT37-1 molecule. The NMR sample was prepared by dissolving 2 mg of compound AT37-1 in 600 µL of CD₃OD. All spectra were recorded on a Bruker Avance 500 spectrometer equipped with a 5 mm triple resonance inverse Z-gradient probe (TBI ¹H, ³¹P, BB). All chemical shifts for ¹H and ¹³C are relative to TMS using ¹H (residual) or ¹³C chemical shifts of the solvent as a secondary standard. The temperature was set at 298 K. All the ¹H and ¹³C signals were assigned on the basis of chemical shifts, spin–spin coupling constants, splitting patterns and signal intensities, and by using ¹H–¹H COSY45, ¹H–¹³C HSQC and ¹H–¹³C HMBC experiments. Gradient-enhanced ¹H COSY45 was realized included 4 scans per increment. ¹H–¹³C correlation spectra using a

gradient-enhanced HSQC sequence (delay was optimized for $^1J_{CH}$ of 145 Hz) was obtained with 56 scans per increment. Gradient-enhanced HMBC experiment was performed allowing 62.5 ms for long-range coupling evolution (320 scans were accumulated). Typically, 2048 t2 data points were collected for 256 t1 increments.

Minimum inhibitory concentrations of AT37-1 compound

The minimum inhibitory concentrations (MIC) of pure active compound were carried out using conventional agar dilution method (Oki et al. 1990) in presence of tetracycline and vancomycin as standard antibiotics. Seven target-*Staphylococcus aureus* were used. The inoculums of these target-organisms were prepared in sterile aqueous suspensions, adjusted by a JASCO V-660 k spectrophotometer to match the turbidity of 0.5 McFarland standard (equivalent to 10^8 CFU/mL) and inoculated by point (1 μ L) on Mueller Hinton medium. This medium contains different concentrations of the active product dissolved in methanol (0.5, 1, 2, 3, 4, 5, 10, 20, 30, 50, 75 and 100 μ g/mL). The antistaphylococcal activity was observed after 24 h of incubation at 30°C. Mueller Hinton medium, with methanol alone and inoculated with target microorganisms was used as a control treatment.

Antibiofilm activity of AT37-1 compound

The biofilms of methicillin-sensitive *S. aureus* MSSA ATCC 25923 and methicillin-resistant *S. aureus* MRSA ATCC 43300 were prepared in 96-well flat-bottom polystyrene microtiter plates using the method of O'Toole (2011) with few modifications as previously described by Stiefel et al. (2016). Both bacteria were firstly grown in 30% tryptic soy broth supplemented with 2.5% glucose (TSBG) at 37°C and 160 rpm. Overnight cultures were diluted to OD_{600 nm} of 0.1 ($\approx 10^8$ CFU/mL) in the same medium. Two hundred microliters of bacterial suspensions were added to each well of a 96-well polystyrene plate. Different concentrations (2, 3, 4, 5, 10, 15, 20 and 25 μ g/mL) of AT37-1 compound, and tetracycline and vancomycin antibiotics were prepared from sterile solutions and added to the respective wells containing the tested bacteria cultures for the determination of the minimum biofilm inhibition concentration (MBIC₅₀). Positive control (cells+TSBG broth) and medium control (TSBG broth) were used as the non-treated control. After incubation at 37°C for 24 h, the biofilm formed in the wells was washed three times with 350 μ L of sterile distilled water. The attached cells (biofilm) were fixed by heating at 60°C for 45 min, then dried, and the wells were stained with 200 μ L of 0.2% crystal violet (CV), and incubated at room temperature for 15 min

after which, the plates were washed three times with sterile distilled water to remove unabsorbed stain. For the quantification of antibiofilm activity, the adherent cells of bacteria associated with crystal violet were solubilized with 95% ethanol and the absorbance was recorded at 595 nm using an ELISA microplate reader (BioTech reader). MBIC₅₀ was defined as the lowest concentration of compound that caused 50% inhibition of the biofilm formation.

Statistics

All experiments were performed independently in triplicate. Values, expressed as means with \pm standard deviations (SD), were compared using one-way ANOVA test (IBM, SPSS-Statistics package, v19 SPSS). The *p*-value ≤ 0.05 was set as significant.

Results

Identification of the strain AT37

Morphological, chemical and physiological characteristics of the strain

The strain AT37 showed very good growth and sporulation on ISP2 and Bennett media, moderate to good growth on ISP4 and ISP3 media and weak growth on nutrient agar medium. The color of aerial mycelium (AM) was grey on ISP2, greyish white on Bennett, ISP3, ISP4 and nutrient agar media. The substrate mycelium (SM) was yellowish brown on ISP2, light yellow on ISP3, ISP4 and Bennett media, and colorless on nutrient agar medium. The micromorphological characteristics showed that the AM produced spiral chains of spores (10–50 spores per chain) carried by sporophores. Endospores, sclerotic granules, synnemata and flagellated spores were not observed. The SM was not fragmented. The strain was not able to produce any pigment on the tested media. Melanoid pigments were also not produced on ISP6 and ISP7 media.

The analysis of cell-wall hydrolysates showed the presence of LL-diaminopimelic acid and glycine. The whole-cell hydrolysates contained non characteristic sugars such as ribose, glucose and galactose. The diagnostic phospholipid detected in its cell membrane was phosphatidylethanolamine.

The physiological properties of the strain AT37 are shown in Table 1. The strain was able to utilize many organic compounds tested, but not arabinose, adonitol, erythritol, mannitol raffinose, phenylalanine, guanine, xanthine and sodium pyruvate. It was able to grow at 4% of NaCl and at 40°C, but it was unable to reduce nitrate and

Table 1 Physiological properties of *Streptomyces* sp. AT37

Characteristics	Result
Carbon source utilization (1% w/v)	
L-Arabinose	–
Adonitol	–
D-Cellobiose	+
Erythritol	–
D-Fructose	+
Galactose	+
Glucose	+
myo-Inositol	+
Lactose	+
Maltose	+
D-Mannitol	–
D-Mannose	+
Melibiose	+
D-Raffinose	–
L-Rhamnose	+
Ribose	+
Sucrose	–
Sorbitol	+
Trehalose	+
D-Xylose	+
Utilization of amino acids (0.1%, w/v)	
L-Proline	+
L-Serine	+
L-Alanine	+
L-Phenylalanine	–
L-Arginine	+
L-Histidine	+
Utilization of organic compounds	
Adenine	+
Starch	+
Esculin	+
Guanine	–
Hypoxanthine	+
Tyrosine	+
Xanthine	–
Gelatine	+
Decarboxylation of organic acid	
Acetate	+
Benzoate	+
Citrate	+
Oxalate	+
Pyruvate	–
Succinate	+
Tartate	+
Resistance to antibiotics (µg/mL)	
Chloramphenicol (25)	+
Erythromycin (10)	+
Kanamycin (25)	–
Penicillin G (25)	–

Table 1 (continued)

Characteristics	Result
Rifampicin (5)	–
Streptomycin (10)	–
Vancomycin (5)	+
Growth with (% w/v)	
Crystal violet (0.05)	–
Phenol (0.001)	–
Sodium azide (0.001)	–
NaCl (4)	+
NaCl (7)	–
Growth at	
pH 5	–
pH 7	+
pH 9	–
30 °C	+
40 °C	+
45 °C	–
Nitrate reduction	+
Production of melanoid pigments	–

Tests: + positive; – negative

to grow in the presence of crystal violet (0.05%), phenol (0.001%) and sodium azide (0.001%), and also at 7% of NaCl, 45 °C, pH 5 and 9. The strain was resistant to chloramphenicol (25 µg/mL), erythromycin (10 µg/mL) and vancomycin (5 µg/mL), but was sensitive to kanamycin (25 µg/mL), penicillin G (25 µg/mL), rifampicin (5 µg/mL) and streptomycin (10 µg/mL). The optimal pH and temperature for growth were 7.0–8.0 and 30 °C, respectively.

Phylogenetic analysis

The 16S rRNA gene of the strain AT37 was sequenced (1472 nucleotides) and has been deposited in the GenBank data library under the Accession No KC414005. The sequence was first analyzed by the EzTaxon-e server and was aligned with those of *Streptomyces* reference species available in the GenBank database. It showed a similarity of 99.6% with *S. novaecaesareae* NBRC 13368 T, the most closely related species. Phylogenetic tree of the strain AT37, which is obtained by applying the neighbor-joining tree, is shown in Fig. 1. It shows its relationship with the type strains of the closest *Streptomyces* species. The strain AT37 constitutes a cluster with *S. novaecaesareae*, the most closely related species.

Antistaphylococcal activity

The activity of the strain AT37 against some multidrug-resistant *S. aureus* on Bennett medium is shown

in Table 2. The strain exhibited an inhibition activity against all tested strains. The inhibition distances were 32, 30, 28, 28, 28, 27 and 26 mm for *S. aureus* ATCC 25923, *S. aureus* R2, *S. aureus* C-MRSA 636, *S. aureus* MRSA 38, *S. aureus* MRSA 39, *S. aureus* VRSA S1 and *S. aureus* MRSA ATCC 43300 strains, respectively. The *S. aureus* ATCC 25923 strain was the most inhibited, while the *S. aureus* MRSA ATCC 43300 was the less inhibited.

Kinetics of growth and production of antistaphylococcal compounds

During the time course of fermentation in Bennett broth medium, production of antistaphylococcal compound, dry cell weight and pH parameters were monitored as shown in Fig. 2. The antistaphylococcal activity was detected at the 2nd day and was maximum at the 5th day of fermentation. The pH kinetics showed variation between 6.0 and 8.0 during the incubation. The maximum dry mycelia weight (10.5 g/L) was reached at 4 days of fermentation.

Extraction of antistaphylococcal compound

The extraction of antistaphylococcal compound was carried out on the 5th day of fermentation corresponding to the optimal production rate. The antistaphylococcal activity was detected on the dichloromethane, acetate ethyl and *n*-butanol extracts against *S. aureus* MRSA ATCC 43300, with diameter of inhibitory zone 16, 11 and 6 mm, respectively. It was not noted on *n*-hexane extract. For that, the dichloromethane was chosen as the best solvent of extraction.

Production and purification of the bioactive compound

The production of antistaphylococcal compound was carried out on the Bennett broth medium. The cell-free supernatant (6.0 L) was extracted by dichloromethane. The yellow organic phase was concentrated to dryness. Bioautography of the resolved dichloromethane extract was realized on TLC and developed in an ethyl–acetate–methanol (100–15 v/v). It showed one active fraction, named ATa ($R_f=0.8$) against *S. aureus* MRSA ATCC 4300. The active fraction ATa, which showed a strong antistaphylococcal activity (30 mm of inhibition diameter), was recovered and desorbed in methanol, concentrated to dryness and used for the final purification by HPLC using a reverse-phase column. The HPLC profiles obtained showed one fraction of antistaphylococcal activity, which was designated AT37-1 (retention time: 39.4 min). This compound was collected and re-injected into the HPLC system until total purification was achieved (Fig. S1).

The pure compound (4.0 mg) presented a white appearance color. It was freely soluble in dichloromethane, methanol, acetone, ethanol, ethyl acetate, water and *n*-butanol, but insoluble in *n*-hexane, benzene and chloroform.

Spectroscopy and spectrometry analysis of the active compound

The absorption spectrum of the purified antistaphylococcal compound AT37-1 in methanol exhibited a maximal absorption at 222, 240 and 284 nm (Fig. 3a). Its IR spectrum exhibited a free and attached hydroxyl group at 3598 cm^{-1} and at 3189 cm^{-1} , respectively, CH, CH₂, CH₃ at 2921 cm^{-1} , C=O at 1723 cm^{-1} , C=C at 1623 cm^{-1} , C–O at $1298\text{--}1,1120\text{ cm}^{-1}$ and HC=CH at $603\text{--}773\text{ cm}^{-1}$ (Fig. 3b). Its mass spectrum showed that it contained an

Fig. 1 Neighbor-joining tree based on 16S rRNA gene sequences showing the position of *Streptomyces* sp. AT37 and its related taxa type species of the genus *Streptomyces*. The numbers at the nodes indicate levels of bootstrap support (values greater than 50%) based on a neighbor-joining analysis of 1000 resampled datasets. Bar, 0.001 substitutions per nucleotide position

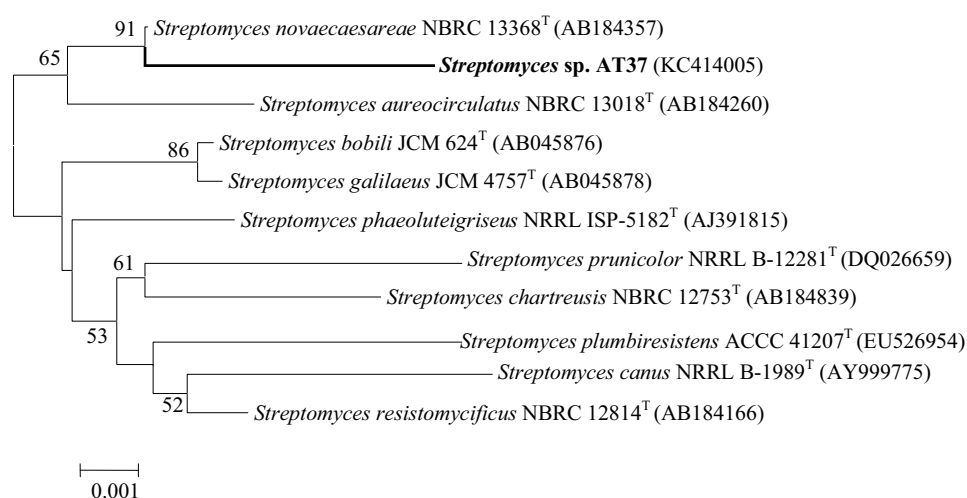


Table 2 Antistaphylococcal activity of *Streptomyces* sp. AT37 against some multidrug-resistant *S. aureus* on Bennett medium

Target staphylococci	Resistance patterns		Inhibition of <i>S. aureus</i> by strain AT37 (in mm) ^a
	Resistance to	Sensitivity to	
<i>Staphylococcus aureus</i> ATCC 25923	None	AN, ATM, CA, FA, GEN, K, OFX, OXA, PEN, TE, TOB, VA	32 ± 1.00
<i>S. aureus</i> MRSA ATCC 43300	AN, ATM, OXA, OFX, PEN, TOB, TE	ATM, CA, FA, GEN, GM, K, VA	26 ± 1.15
<i>S. aureus</i> C-MRSA 636	AN, ATM, GEN, K, OFX, OXA, PEN, TOB, TE	CA, FA, VA	28 ± 1.00
<i>S. aureus</i> MRSA 38	FA, K, OXA, PEN, TE	AN, ATM, CA, GEN, OFX, TOB, VA	28 ± 0.76
<i>S. aureus</i> MRSA 39	K, OXA, PEN, TE	AN, ATM, CA, GEN, FA, OFL, TOB, VA	28 ± 1.00
<i>S. aureus</i> S1	CA, GEN, K, PEN, VA	AN, ATM, GEN, FA, TE, OFX, OXA, TOB	27 ± 0.76
<i>S. aureus</i> R2	PEN	AN, ATM, CA, GEN, FA, K, OFX, OXA, TE, TOB, VA	30 ± 1.00

AN amikacine, ATM aztreoname, CA clindamycin, FA fusidic acid, GEN gentamicin, K kanamycin, OFL oleandomycin, OFX ofloxacin, OXA oxacillin (methicillin), PEN penicillin-G, TE tetracycline, TOB tobramycin, VA vancomycin

^aAverage ± standard deviation from three replicates per treatment

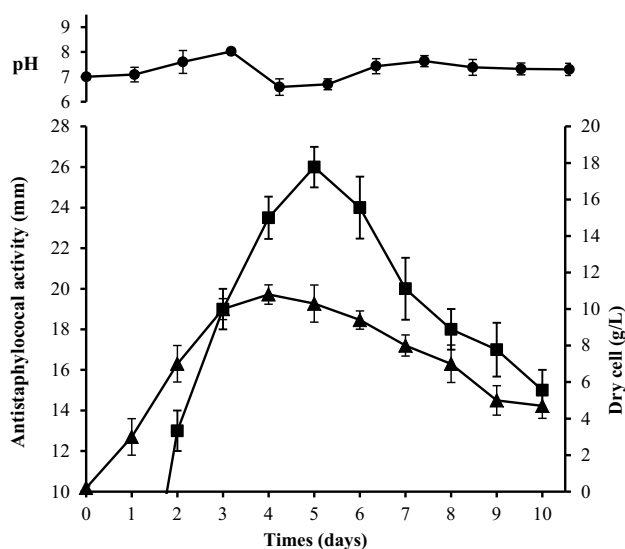


Fig. 2 Time course of pH, growth and antistaphylococcal compound production by *Streptomyces* sp. AT37 on Bennett broth medium against *S. aureus* MRSA ATCC 43300 Antistaphylococcal activity (filled square); dry cell (g/L) (filled triangle); pH (filled circle). Measurements of activity against bacterium represent the diameters of inhibition with including the diameter of wells (10 mm). Each measure represent: average ± standard deviation from three replicates per treatment

ion peak at m/z 393.5 (M-H) (Fig. 4). Thus, the molecular weight of this compound was $M = 394.4$.

The ^1H and ^{13}C chemical shifts of the compound AT37-1 are given in Table 3 and its structure can be seen in Fig. 5a. The ^{13}C , HSQC and HMBC spectra show 22 carbon signals for this compound. It was possible to discern one ketone group (δ_c 202.14), 5 alcohol groups

(δ_c from 67.18 to 103.28), 9 sp^3 -hybridized carbons (δ_c to 39.33) and 8 sp^2 -hybridized carbons (δ_c 110.30 to 186.25). The hydrogens of the hydroxyl groups are not observed due to rapid exchange with MeOD. The 2D ^1H - ^1H and ^1H - ^{13}C experiments and especially the long range ^1H - ^{13}C couplings observed in the HMBC spectrum (see Fig. 5b) permitted to establish the connectivity between all the groups of the molecule.

The structure of the AT37-1 compound was determined by NMR and mass spectrometry to be 5-[(5E,7E,11E)-2,10-dihydroxy-9,11-dimethyl-5,7,11-tridecatrien-1-yl]-2-hydroxy-2-(1-hydroxyethyl)-4-methyl-3(2H)-furanone, which is known in literature as antibiotic E-975.

Minimum inhibitory concentrations

Minimum inhibitory concentrations (MIC) of the AT37-1 compound in comparison with tetracycline and vancomycin as standard antibiotics are summarized in Table 4. MIC values against target staphylococci were comprised between 15 and 30 $\mu\text{g/mL}$. The MIC values of the AT37-1 compound was 15 $\mu\text{g/mL}$ against *S. aureus* ATCC 25923 and *S. aureus* R2, 20 $\mu\text{g/mL}$ against *S. aureus* MRSA 636, *S. aureus* MRSA 38, *S. aureus* MRSA 39 and *S. aureus* VRSA S1, and 30 $\mu\text{g/mL}$ against *S. aureus* MRSA ATCC 43300. However, both standard antibiotics showed MIC values ranging between 5 and 20 $\mu\text{g/mL}$ for vancomycin, and 15 and 30 $\mu\text{g/mL}$ for tetracycline against target staphylococci. The most sensitive strains were *S. aureus* ATCC 25923 and *S. aureus* R2.

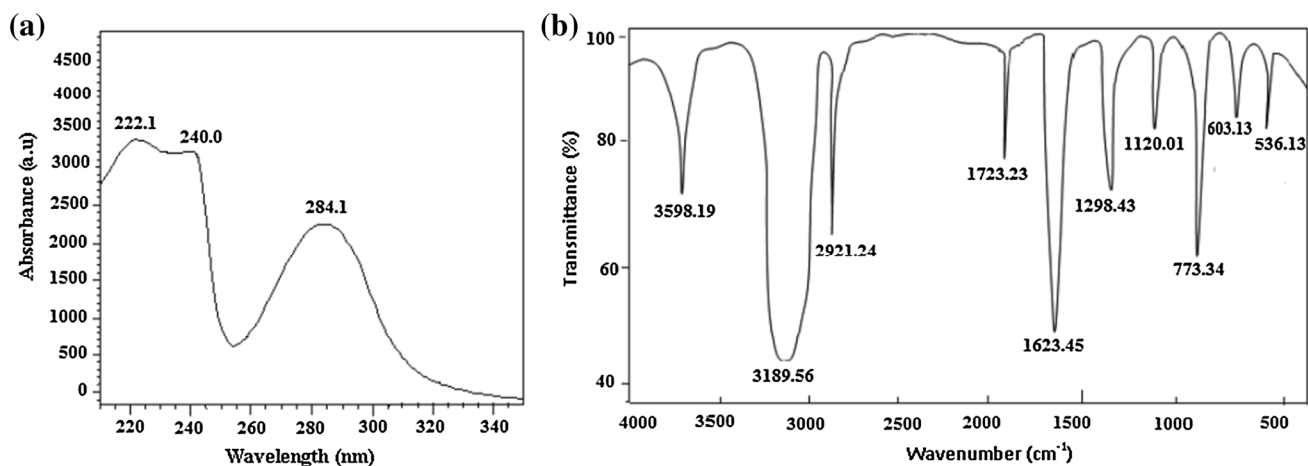


Fig. 3 UV-Vis (a) and IR (b) spectra of AT37-1 compound produced by *Streptomyces* sp. AT37

Antibiofilm activity of AT37-1 compound

In order to determine the minimum biofilm inhibition concentration (MBIC₅₀), several concentrations which are less than those of AT37-1 compound, tetracycline

antibiofilm activity against methicillin-sensible *S. aureus* MSSA ATCC 29523 and methicillin-resistant *S. aureus* MRSA ATCC 43300. Compared to the positive control, the AT37-1 compound showed a significantly reduction in the biofilm formation by MRSA and MSSA at the

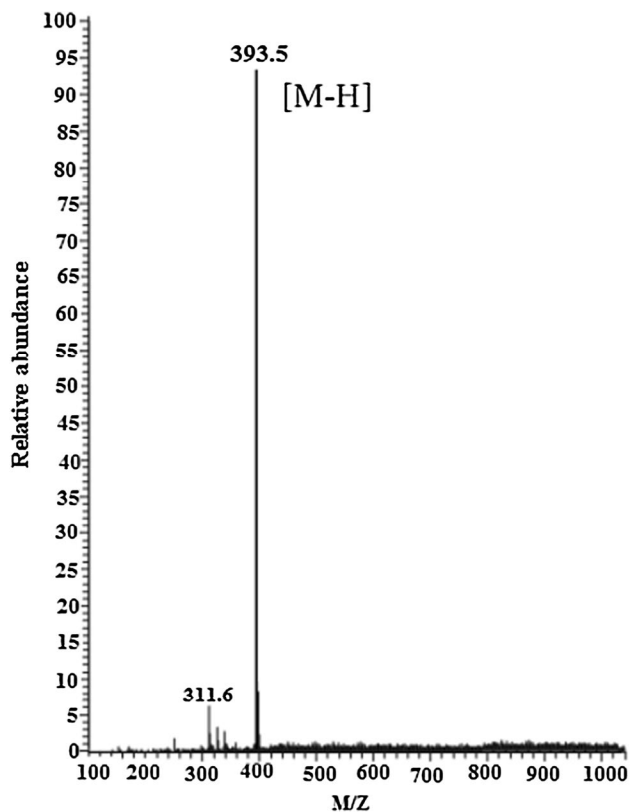


Fig. 4 Mass spectrometry profile of AT37-1 compound by electro spray ion source in positive mode

Table 3 ¹H and ¹³C NMR data assignments of AT37-1 compound in CD₃OD at 298 K

¹ H and ¹³ C number	¹ H chemical shift (ppm)	¹³ C chemical shift (ppm)
1	0.82	8.81
2	4.64	120.62
3	–	135.76
4	0.83	10.90
5	2.84	81.22
6	1.52	39.33
7	0.04	15.63
8	4.78	134.17
9	5.27	129.46
10	5.28	130.22
11	4.81	130.23
12	1.40–1.47	27.53
13	0.83–0.87	35.87
14	3.27	67.18
15	1.93–1.97	36.05
16	–	186.25
17	–	202.14
18	–	103.28
19	–	110.3
20	0.87	3.5
21	3.11	67.85
22	0.50	14.46

and vancomycin antibiotics were used to evaluate the

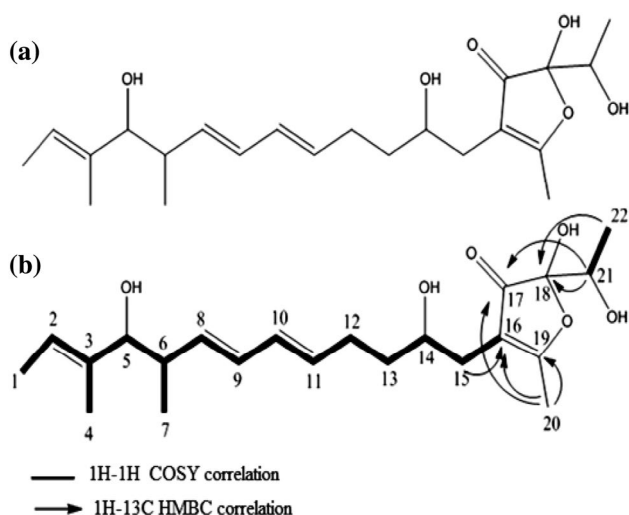


Fig. 5 Structure of the compound AT37-1 (a) and COSY and HMBC correlation (b)

Table 4 Minimum inhibitory concentrations (MIC) of the active compound AT37-1 produced by *Streptomyces* sp. AT37 against staphylococci strains

Target staphylococci	MIC ($\mu\text{g/mL}$) ^a		
	AT37-1	Tetracycline	Vancomycin
<i>Staphylococcus aureus</i> MSSA ATCC 25923	15	15	5
<i>S. aureus</i> MRSA ATCC 43300	30	30	10
<i>S. aureus</i> MRSA 636	20	30	10
<i>S. aureus</i> MRSA 38	20	30	10
<i>S. aureus</i> MRSA 39	20	30	10
<i>S. aureus</i> S1	20	15	20
<i>S. aureus</i> R2	15	15	5

^aMIC values represent the average of two repetitions

MBIC₅₀ of 15 and 10 $\mu\text{g/mL}$, respectively, corresponding to $\approx 1/2$ MICs. However, the tetracycline and vancomycin antibiotics exhibited MBIC₅₀ values from 20 to 15 $\mu\text{g/mL}$ and from 10 to 4 $\mu\text{g/mL}$ against MRSA and MSSA, respectively, as shown in Fig. 6.

Discussion

The strain AT37 had a good growth on the culture media tested. It produced a grey aerial mycelium forming spiral spore chains, and non-fragmented substrate mycelium. Its whole-cell hydrolysates contained LL-diaminopimelic isomer and glycine, and non characteristic sugar such as ribose, glucose and galactose, typical of cell-wall type IC (Becker et al. 1964; Lechevalier and Lechevalier 1970).

The cell membrane contained phosphatidylethanolamine as characteristic lipid, corresponding to phospholipid type PII (Lechevalier et al. 1977). According to the morphological and biochemical characteristics, the strain AT37 was attached to the genus *Streptomyces* (Holt et al. 1994), which currently contains 615 species (Labeda et al. 2012).

The 16S rRNA gene sequence of the strain AT37 confirmed its membership to genus *Streptomyces*. The strain sequence was compared with those of other *Streptomyces* species. The similarity level was 99.6% with *S. novae-caesareae* NBRC 13368 T, the most closely related species (Labeda et al. 2012). Indeed, much higher 16S rRNA gene similarities have been found between representatives of validly described *Streptomyces* species, such as the type strains of *S. indigoferus* and *S. herbaricolor* (100%), *S. rangoonensis* and *S. coeruleus* (100%), *S. bellus* and *S. wer-raensis* (100%), and *S. janthinus* and *S. parvulus* (99.9%) (Kämpfer 2012). Contrary to our strain AT37, *S. novae-caesareae* does not produced an aerial mycelium on any of the ISP media and its substrate mycelium was greyish yellow to greyish olive on ISP2 medium. It utilized arabinose, mannitol, sucrose and raffinose for growth (Kämpfer 2012). Moreover, *S. novae-caesareae* has not been reported to produce any bioactive compound. Based on these results, the strain AT37 could be a new species, but the DNA–DNA hybridization is necessary to confirm this result.

The strain AT37 showed a strong activity against some strains of *S. aureus* especially methicillin resistant (MRSA) and vancomycin resistant (VRSA) *S. aureus*. Many strains and species of *Streptomyces* have been reported to produce bioactive compounds with diverse biological activities and chemical structures (Solecka et al. 2012). Some of these strains were isolated from soil and showed anti-*S. aureus* activity (Aouiche et al. 2012), but few of them revealed activity against multidrug-resistant *S. aureus* (Driche et al. 2015).

One antistaphylococcal metabolite, named AT37-1 is produced by our strain in Bennett's medium. It was subjected to several analyses to determine its chemical structure. On the basis of UV–Visible, IR data, NMR, and mass spectrometry, its structure was identified to be 5-[(5E,7E,11E)-2,10-dihydroxy-9,11-dimethyl-5,7,11-tridecatrien-1-yl]-2-hydroxy-2-(1-hydroxyethyl)-4-methyl-3(2H)-furanone. This compound is known as antibiotic E-975 belonging to the group of furanone and the heterocycles family, which includes several natural products and drugs with diverse biological activities (Fujiwara et al. 2015). The antibiotic E-975 was isolated only from *Streptomyces* sp. Eco86 (Banskota et al. 2006) and *S. gramineus* IR087Pi-4 (Fujiwara et al. 2015). This antibiotic has been shown moderate cytotoxic activity with IC₅₀ values of 2.3 μM (Fujiwara et al. 2015) and electron transport enzyme inhibitory activities against *Ascaris suum*

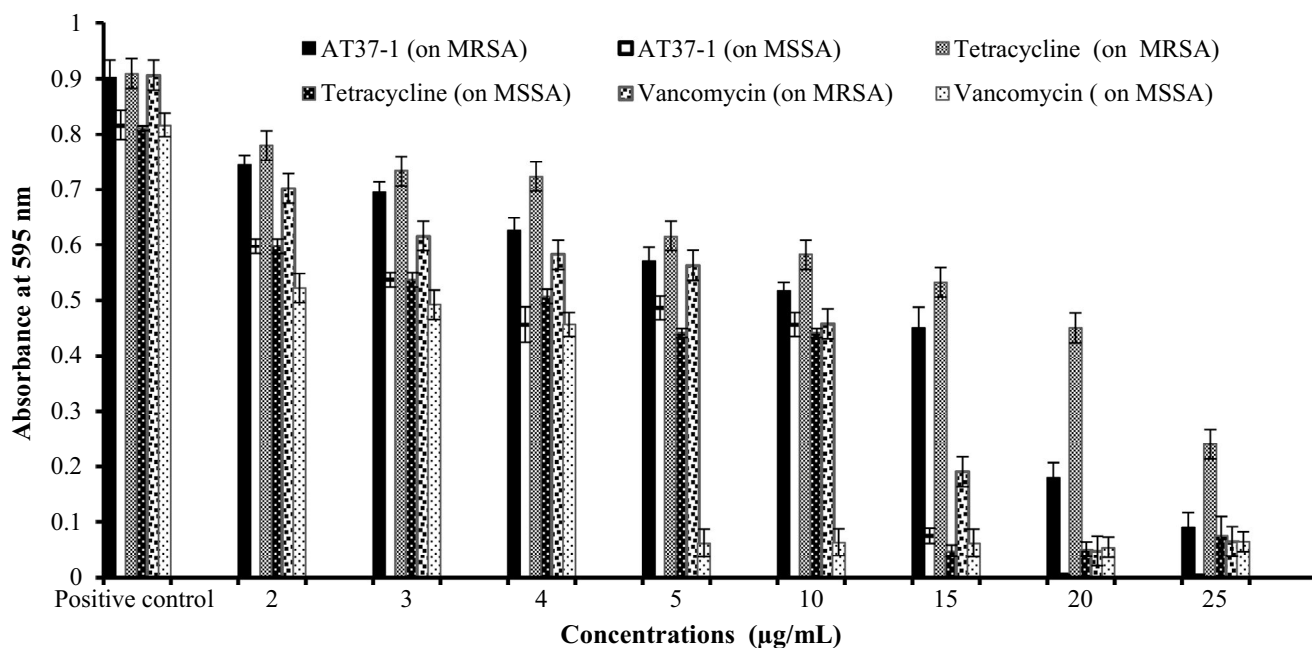


Fig. 6 Effect of AT37-1 compound on the biofilm formation by methicillin-sensitive *S. aureus* MSSA ATCC 29523 and methicillin-resistant *S. aureus* MRSA ATCC 43300. The optical density of stained adherent bacteria with crystal violet was determined with a 96 microplate reader at 595 nm (OD). The results are expressed as average optical density readings for crystal violet assays compared

to positive control. Results showed the average of three independent experiments. Statistically significant differences were performed for each concentration as determined by Student's t test, $P < 0.05$, compared to biofilm formation in the absence of the AT37-1 compound, and tetracycline and vancomycin antibiotics

NADH-fumarate reductase (Banskota et al. 2006). However, its antibacterial activity has not been reported in the literature. On the other hand, many other furanone derivatives have been isolated from species of *Streptomyces*, such as 5-alkenyl-3,3(2H)-furanone (E-837) from *Streptomyces aculeolatus* NRRL 18422 and E-492 derivative from strain *Streptomyces* sp. Eco86 (Banskota et al. 2006).

The antibiotic AT37-1 exhibited interesting antistaphylococcal and antibiofilm activities by comparison to tetracycline. However, this antibacterial activity was lower compared to vancomycin. The biofilm formation was reduced by 50% at a concentration of 10–15 µg/mL. Several furanone derivatives have been reported in the literature as antibiofilm molecules (Hentzer et al. 2002; Trizna et al. 2016). Among them, halogenated furanones, isolated from marine red algae (*Delisea pulchra*), or synthesized furanones showed activity against both Gram-positive and Gram-negative bacteria, including an inhibitory effect on biofilm formation for one of these compounds. However, the use of halogenated furanones is limited due to their toxicity in mammalian cells (Hentzer et al. 2002; Ren et al. 2004). 2(5H)-furanone derivatives which were synthesized chemically, and with no antibacterial activity, inhibited the biofilm formation of *S. aureus* at concentrations of 10 µg/mL (Trizna et al. 2016). However, the antibiotic AT37-1 showed both interesting antistaphylococcal and antibiofilm

activities. These results are interesting owing to the biofilm formation and the resistance of *S. aureus* to many antibiotics used in therapeutic treatment.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interests regarding the publication of this paper.

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